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Original article

# Comparative molecular characterization of three *Diplozoon* species from fishes of Kashmir Valley

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ABSTRACT

Present study reports the results of molecular analysis of the internal transcribed spacer (ITS) of ribosomal DNA of 3 Monogenean species using polymerase chain reaction (PCR), nucleotide sequencing and construction of phylogenetic trees from different fish hosts of Kashmir. The present study shows that the size of the amplified product is 873 bp long for *D. kashmirensis*; 1120 bp long in *D. aegyptensis* and 687 bp long in *D. guptai* revealing that there are intraspecific differences in their base pair lengths. Guanine and Cytocine (G+C) content of three *Diplozoon* species was found nearly constant for three species i.e., 47% (*D. kashmirensis*); 47% (*D. aegyptensis*) and 48% (*D. guptai*), this GC richness contributes to physical attributes of RNA structures, as there is correlation between GC content and optimal growth temperature. An important observation during the present study has been noticed that *Schizothorax niger* is infected by all the three species of *Diplozoidae*: *D. kashmirensis*; *D. aegyptensis* and *D. guptai*, but when all six fishes were collected simultaneously, parasitism by all the parasite species was never observed. Phylogenetic trees Maximum Parsimony (MP), Maximum Likelihood (ML) and Neighbor Joining (NJ) showed that *D. kashmirensis* and *D. aegyptensis* share a common host *Carassius carassius* and *S. niger*.

## 1. Introduction

Monogeneans belonging to the Diplozoidae are common parasites on the gills of cyprinid fish. The life cycle is direct, including free-swimming oncomiracidia, larval stage (diporpa), and adult. Two larvae (diporpae) permanently fuse into a pair to form the sexually maturated adult. In the adult, the vitellaria and almost all the internal organs are situated in the anterior part of the body. The female and male reproductive organs and terminal part of the gut are situated in the posterior part. The attachment apparatus of adults consist of four pairs of clamps and a pair of small central hooks situated on the ventral side of the opisthaptor. Due to the complicated determination of several groups of monogenean parasites, molecular markers based on species-specific variability in the ribosomal DNA region (rDNA) their cytogenetics have been designed and shown to be useful for precise species identification (Cunningham 1997; Matejusova et al. 2001a; Huyse and Volckaert 2002; Zietara et al. 2002; Simkova et al. 2006). The interspecific nucleic acid variability of internal transcribed spacers of rDNA (ITS) has also been used to distinguish diplozoid parasites (Matejusova et al. 2001b; Sicard et al. 2001; Matejusova et al. 2002; Sicard et al. 2003; Matejusova et al. 2004; Gao et al. 2007).

From the available data, it has been concluded that morphological and metrical differences in the clamp size, pharynx size, prohaptoral length, opisthohaptoral length, sucker distance, testis, ovary and egg size were the major criteria for species determination. Species deterimination of trematodes is difficult and demands great skill and experience. As the structures of taxonomic importance (central hooks, clamps etc) grow gradually and the measurements of sclerotized structures are variable, species determination of trematodes in different developmental stages is not always clear. There are still some unclear descriptions of trematode species that differ only by host species, and some studies that did not employ recommended criteria (Jiang et al., 1985; Kritscher, 1991). Molecular biology techniques have been used as objective methods to distinguish between parasite species. The rDNA genes, particularly the 28S gene, have been found generally useful in molecular taxonomy and phylogeny of parasites (Blair & Barker, 1993; Cunningham et al., 1995; Zhu et al., 1998). However, there are no published molecular studies of trematode genomes from the Kashmir valley. The present study reports the results of molecular analysis of the internal transcribed spacer (ITS) of ribosomal DNA of 3 Monogenean species namely Diplozoon kashmirensis Kaw, 1950; Diplozoon aegyptensis Fischthal et Kuntz, 1963; Diplozoon guptai Fayaz and Chishti, 2000 using polymerase chain reaction (PCR), nucleotide sequencing and construction of phylogenetic from different fish hosts of the Kashmir valley.

## 2. Materials and methods

### 2.1. Parasite Material

Parasite specimens of Diplozoon spp. were collected from the Carassius carassius; Cyprinus carpio communis; C. c. specularis; Schizothorax niger; S. esocinus; S. curvifrons and S. plagiostomus of Kashmir and were used for DNA extraction. Samples were immediately fixed in 70% alcohol after collecting from the gills, gill cover, mouth cavity, eyes & fins of host fish. These samples were remained in alcohol until the present study.

### 2.2. DNA isolation

Phenol-Chloroform Technique: The detailed protocol is as follows (Sambrook and Russell, 2001):

Wash the parasite material in PBS.

↓

Place in a 1.5 ml eppendorf tube (UV cross- linked), grind with a pestle (UV treated) and add 300 µl of NET buffer.

↓

Add 15 µl SDS and 6 µl Protinase-K (10 mg/ml); store at -20$^0$C.

↓

Incubate homogenate at 37$^0$C overnight.

↓

Add 400 µl of 25:24:1 phenol/chloroform/isoamyl alcohol (stored at 4$^0$C); mix the contents gently (~2 minutes).

↓

Centrifuge at 12,000 rpm for 10 minutes.

↓

Pipette out the upper phase (~280 µl) into a fresh 1.5 ml tube.
↓
Add 300 µl of 24:1 chloroform/isoamyl alcohol (stored at room temp.) to the sample; mix gently (~2 minutes).
↓
Centrifuge at 10,000 rpm for 10 minutes.
↓
Remove upper aqueous phase to a fresh 1.5 ml tube.
↓
Add 1 ml of ice-cold 100% ethanol (stored at -2$^0$C) to the aqueous solution; mix gently; store at -2$^o$C for 1 hour.
↓
Centrifuge at 10,000 rpm for 10 minutes (DNA will precipitate).
↓
Remove supernatant.
↓
Wash the pellet in 1ml 70% ethanol at 10,000 rpm for 10 minutes.
↓
Tip out the alcohol after the spin.
↓
Dry DNA pellet; resuspend in mili-Q water, store at 4$^o$C.

**Table 1**
Primers used for Trematodes.

| Species | Primer Designed | GenBank Accession Number | Author and Year |
|---|---|---|---|
| *Diplozoon kashmirensis* Kaw, 1950<br>*Diplozoon aegyptensis* Fischthal *et* Kuntz, 1963<br>*Diplozoon guptai* Fayaz and Chishti, 2000 | Forward<br>Cer5.8S 2249:5/GCTCACGTGACGATGAAGAG3/<br>Reverse<br>Cer28S 3116 :5/TTCGCTATCGGACTCGTGCC3/ | AF 369758 to AF 369761 | Sicard *et al*., 2001 |

[**Reagents for PCR:** Taq DNA polymerase 3U/µl, dNTP mixture 100mM, primers 20 pmols, 10xTaqDNA Polymerase buffer (Genei), PCR water (Sterile milli-Q)].

**Sequences deposited in GenBank**
GenBank: AF973616; *Diplozoon kashmirensis*, complete sequence.
GenBank: AF973617; *Diplozoon aegyptensis*, complete sequence.
GenBank: AF973618; *Diplozoon guptai*, complete sequence.

## 3. Results

The three monogenean species of Trematodes viz., Diplozoon kashmirensis Kaw, 1950; Diplozoon aegyptensis Fischthal et Kuntz, 1963 and Diplozoon guptai Fayaz and Chishti, 2000 which were recovered during the present study are used for molecular study for the first time as under;

**(a) Extraction of DNA**

Parasite specimens of three Diplozoon species were collected from fish hosts of Carassius carassius; Cyprinus carpio communis; Schizothorax curvifrons; Schizothorax esocinus; Schizothorax niger and Schizothorax plagiostomus from Wular lake; Anchar lake; Dal lake; Manasbal lake; River Jhelum and River Sindh of Kashmir valley, preserved in 100% ethanol for genomic DNA extraction and stored at -200C for good quality of DNA. For DNA extraction ethanol was removed from parasites as per the protocol given in methodology and as such, these specimens were air dried to remove ethanol. The resultant DNA was examined on 1.5% agrose-TAE gels, stained with ethidium bromide (EtBr) and visualized under UV light.

**(b) PCR amplification**

The PCR amplified products of ITS regions of rDNA were successfully obtained using the primers as mentioned in Material and Methods. PCR amplification was carried out to amplify ITS region of Diplozoon species (Table 2). The size of the amplified product was found to be 873 bp long for D. kashmirensis; 1120 bp long in D. aegyptensis and 687 bp long in case of D. guptai (Figs. 1-4). In BLAST search of these sequences, they showed similarity with other Diplozoon spp. (Table 3). In bioinformatics analysis, the results tallied with those of the earlier study; hence, the same are not repeated herein. Based on morphological studies, these species were identified as belonging to three Diplozoon species. The present results of the molecular analysis corroborate the species identification of these forms. Therefore, it can be assumed that the present species recovered from the different fish hosts of water bodies of Kashmir valley is D. kashmirensis Kaw, 1950; Diplozoon aegyptensis Fischthal et Kuntz, 1963 and Diplozoon guptai Fayaz and Chishti, 2000.

**Table 2**

PCR assay of Monogeneans which were carried out in a thermocycler (Eppendorf Mastercycler Personal) under different conditions.

| Monogenea | Initial Denaturation | Denaturation for 30 cycles | Annealing | Extension | Final extension |
|---|---|---|---|---|---|
| *Diplozoon kashmirensis*; *D. aegyptensis* and *D. guptai* | 95°C for 10 minutes | 30 cycles at 95°C for 30 seconds | 55°C for 30 seconds | 72°C for 75 seconds | 72°C for 10 minutes |

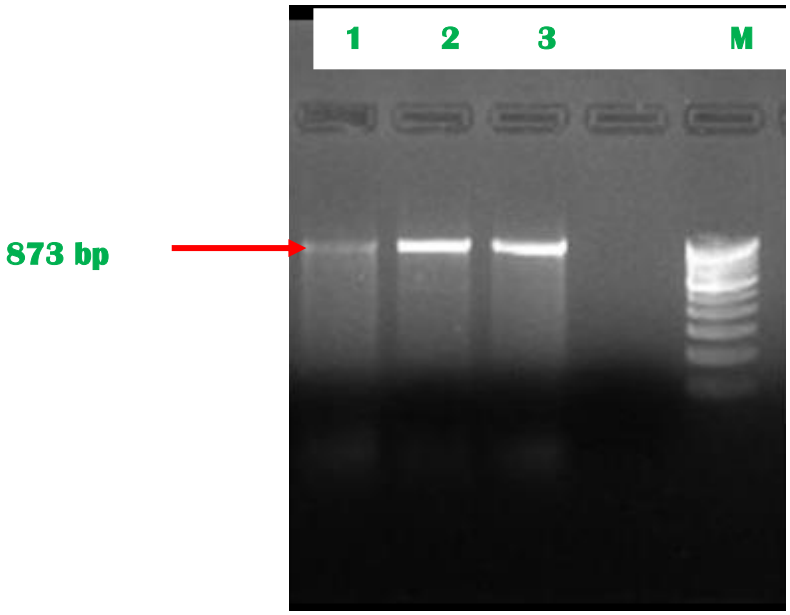


**Fig. 1.** PCR product of *Diplozoon kashmirensis* Kaw, 1950, M = marker; bp = base pairs (100 bp ladder).

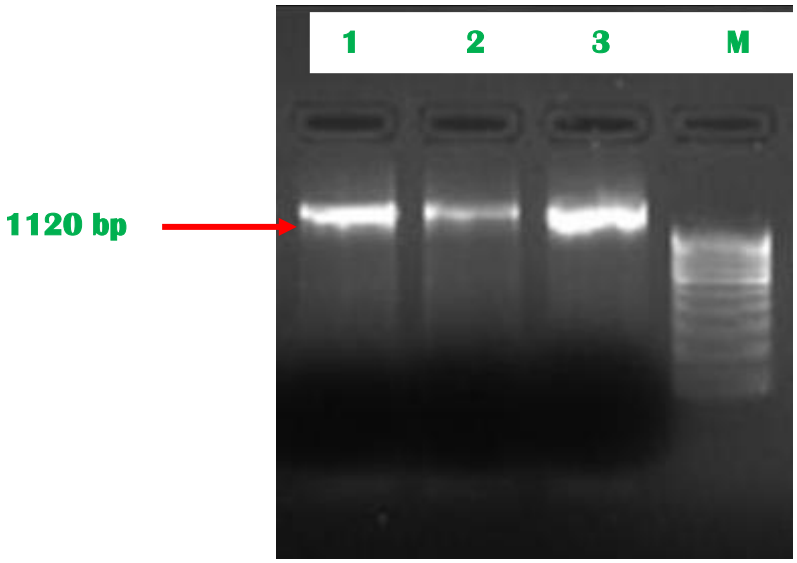


**Fig. 2.** PCR product of Diplozoon aegyptensis Fischthal et Kuntz, 1963, M = marker; bp = base pairs (100 bp ladder).

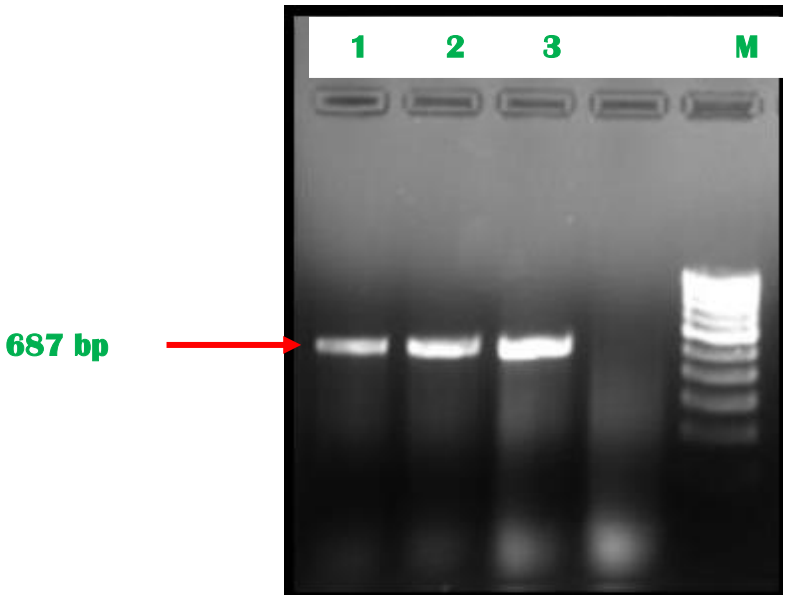


**Fig. 3.** PCR product of Diplozoon guptai Fayaz and Chishti, 1999, M = marker; bp = base pairs (100 bp ladder).

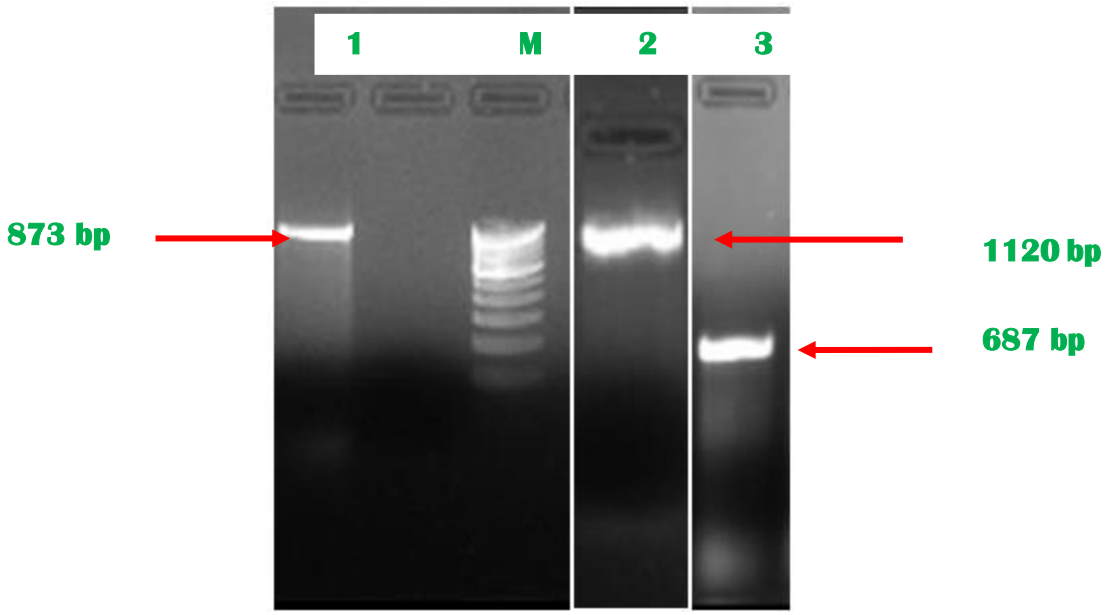


**Fig. 4.** Polymerase Chain Reaction (PCR) products of Trematodes (Monogenea) M = marker; bp = base pairs (100 bp ladder), 1 = Diplozoon kashmirensis Kaw, 1950, 2 = Diplozoon aegyptensis Fischthal et Kuntz, 1963 and 3 = Diplozoon guptai Fayaz and Chishti, 1999.

**(c) Nucleotide sequences**

PCR products were visualized and documented, and the sizes of the sequences were estimated. The sequence obtained from three different Diplozoon species were submitted to GenBank and their accession number acquired were AF973616; AF973617 and AF973618 (Table 4). Sequences were compared with other sequences of monogenean species from GenBank. When the BLAST search was performed, the query sequence showed maximum similarity with 28S rDNA sequence of Diplozoon spp. The nucleotide sequences obtained and shown in Figs. 5-7 are as raw sequences (Tables 5-7).

```
  1 TGCTTACTGA CTTGAGCATC GATTTCTTGA ACGTGAATTG CGGCATTACC CTCTAATGAT
 61 GCCACGCCTA GCCGAGTATC GGCATTAAAT CTAGCACGAC GCTTATTTGG TCCTGGCTTA
121 GAAAGTTGTC AGCCGTCGTG TTGTACTTGG CAACGTGTTG TTCTGTTGTC AAGTCGGCGG
181 TATTATTGAC GCTTGCCAAA TGTAATGGAG AGTTTGTATA TGCGAAATAT CTTCCGGTAG
241 CCTGTTGGTG TTGGCTACGC TGCCCCGTGT ATTTTTTATT TGCATTTTTG TGCATACCGA
301 TGGGGTGGTT AGCTTCTCGT CAGCAGTGCG TCCTTGCCGG TGGTGTCGTG GAATGGGAAT
361 TTCAATAAGC ATTTCTGAAT CCTAATTGTG AAATTGTCAT TTTATGTGCT GTTCTCTTGA
421 GCCGCATGGC CCACTTGTTG TGCGATGACC AGTGACGCTT TGAATGCGAG TGCATGCATG
481 CCAGGTCTCA GCCTATTTGT GATCGCGACA GTGCTTTGCT TGTGTTCTGC GTTTAATTTT
541 TGTCACTGTT TCCCGCGAAT GAGCGAGTCT GGCCCGAGAC GAGAGCATGT GCCCATGTCG
601 TGCTGTGCAG ACATTACTAC TCCATTCTTC GCTAAGTGTG TATCGGTGTC ACCCGTATTT
661 TACTGTACTT CTGTGGTGTA TGCACCTGAC CAAGGATTAG GCGTGATCAC CCGCTGAGCT
721 TAAGCATATC AATGGGCGGA GGAAAAGAAA CTAACCACTA TTCCCTTAGT AACGTCGAGT
781 GAACACCGAT TAGCAAAGCA CCGAAGCTGC GGTCTTTTGG CCGTTCGGCA ATCCGGTGTT
841 TAGGTTATCA TACTCAGGCG ATGTACTGTG GTC
```

**Fig. 5.** Raw nucleotide sequences of Diplozoon kashmirensis Kaw, 1950.

```
  1 AACTGCAAAC TGCCTTGAGC AAATTAGTTG TGAAAGTAAA TTACGGCAGG AGGCTCCCCC
 61 TGATAACACG CCTAGCCCCG TGTCGGCATT AAATCGATCA CGACGCTTAA TTGGTTGTGG
121 CTTAGTTTGT TGTCAGCCGT CGTGTTGTAC TTGGCAACGT GTTGTTCAGT TGTCAAGTAG
181 ACGGTATTAT TGACGCTTGC CAAATGTAAT GGAGAGTTAG NDATGCGAAA TATCCGCTGG
```

```
 241 TAGCCTGTTG GTGTTGGCAA CGCTGGCCCG TGTATGGTTT ACTTGTTTTT TTGTGCATAC
 301 TCATGGGGGC GGTTAACTTC GCGTCATCAG AGCGTGTTTG CCGGAAGTGT ATTGCAGTGG
 361 CGTGGGAATT TCAATGAGCA TTTGTGAATG GTAATTGTTA AATTGCCATT TTATGTGCTG
 421 TTCTCTTGAG CCTTTTGGCC CACGGGTTGT GCGGTGACCA GTGTTGCTTT GAATGCGTGC
 481 GCATGCATGC CAGGTCGCAG CCTATTGTGA TCGCGACAGT GCTTTGCTTG TGTTCTGCGT
 541 TTAATTTTTG TCACTCCCGC ACTGGTCGCT AAGTGCATGT CCCGAGATGA GATTGTGTGC
 601 CCATGTCATG CTGGGCTGAC ATTACTACTC CACTGGTCGC TAAGTGCATG TCGGTGTCAT
 661 CAGTATTCTA CTGTACTGCT GTGTTGTGTG TGCACCTGAC CTCGGATTAG GCGTGATTAC
 721 CCGCTGAACT TAAGCATATC AATAAGCGGA GGATTAGAAA CTAACCAGGA TTCCCTTAGT
 781 AACGGCGAGT GAACAGGGAT TAGCCCAGTT CCGAAGCTGC GGTCTTTTGG CCGTTCGGCA
 841 ATGTTATGTT TAGGTTGGCA TACTCAGGCG ATGTACTGTG CTAAGTCCAT TCATGAATAT
 901 GGCTAGCTAT CTGTTCCAGA GAGGGTGAAA GGCCCGTGAG CATAGTACGT TGTTCTGTCT
 961 TAGCCAACCG TTGAGTCGGG GGTTTACTTG AGGCAGCCCA AAAAGTAGAC GGTATTATTG
1021 ACGCTTGCCA AATGTAATGG AGTTAGTGTG ACCCGAGATG AGATTGGTTG GCATACGCAG
1081 GCGATGTACT GTGCTAAGTC CAGGTGTTTG CATTATTAGT
```

**Fig. 6.** Raw nucleotide sequences of Diplozoon aegyptensis Fischthal et Kuntz, 1963.

```
   1 TGCTGCAAAC TGCCTTGAAA ATCTTCTTCT TGAACGCGAA TCGCGGTATT AGGTACTGCC
  61 TGATGCCACG CCTAGCCGAG TGTTGGCATT ATATCTATCA CGACGCTTAA TTGGTCGTGG
 121 CTTAGGCGGT TGTCCTCCGT CGTGTTTTAC TTTGCAACGT GTTGCTCAGT TGTACTGTCG
 181 ACGGTATTAT TGACGCTTGC CAAATGTAAT GGAGAGTGTG TATATGCGAA ATTTCTGCCG
 241 GTAGCCTGTT GGCTGCGGCG ACGCTGCCCC GTGGCCGGTT TACTTGCATT TTTGTATCTA
 301 CCGATTGGGG CGGTTAGCTT GTATTCATCA GCCCGTGTTT GCCGGTGGTG ACTCGTGGTG
 361 GCGTGGGAAT TTCAATAAGC ATTACTGAAT GGTAATTAAT AAATTGCCAT TATATATGCT
 421 GTGCGCTTGA GCCTTTTGGC CCACGGGTTG TATTGTGACC AGTGTTGCTT TGAATGCGCT
 481 CGCAAGCATG CCAGGTCTCA GCCTATGGTG ATCGAGACAG TTCTTTGCTT GTGTTATGCG
 541 TTTAGGTGTT GTCACCTCTA CTTGCATATG TGCTAGTGTG TACGCGGAAT GAGCTTTTGT
 601 GCCCATGTCA TGCTGTGCTG ACGCTACTTC TCCACTGGTC CAGAAGTGCA TGTCGGGGTC
 661 ACCATAACTT TGCTGTATTG TGGGTGC
```

**Fig. 7.** Raw nucleotide sequences of Diplozoon guptai Fayaz et Chishti, 2000.

From Tables 5-7, it can be concluded that the three species of Diplozoons have different base base lengths & total number of amino acids and have nearly same G+C content, therefore they are equally stable at higher temperature.

**(d) Pairwise alignment**

Pairwise alignments of Diplozoon species were made by using different softwares as discussed in materials and methods. D. kashmirensis showed maximum similarity with those of D. bliccae where as D. aegyptensis showed maximum similarity with D. paradoxum and in case of D. guptai that showed maximum similarity to D. homoin as shown in the following Tables 8-10.

**(e) Construction of phylogenetic tree**

Phylogenetic trees were obtained by comparing the 28S rDNA sequences of the query parasite and other available sequences for related monogenean parasites. The E value was found to be zero up to the 100th sequence of BLAST search and the query coverage 95% and above (Table 3). The species of D. kashmirensis and D. aegyptensis appeared to be the most closely related species, with well-supported clade by Neighbour joining and MP trees (Figs. 8-10).

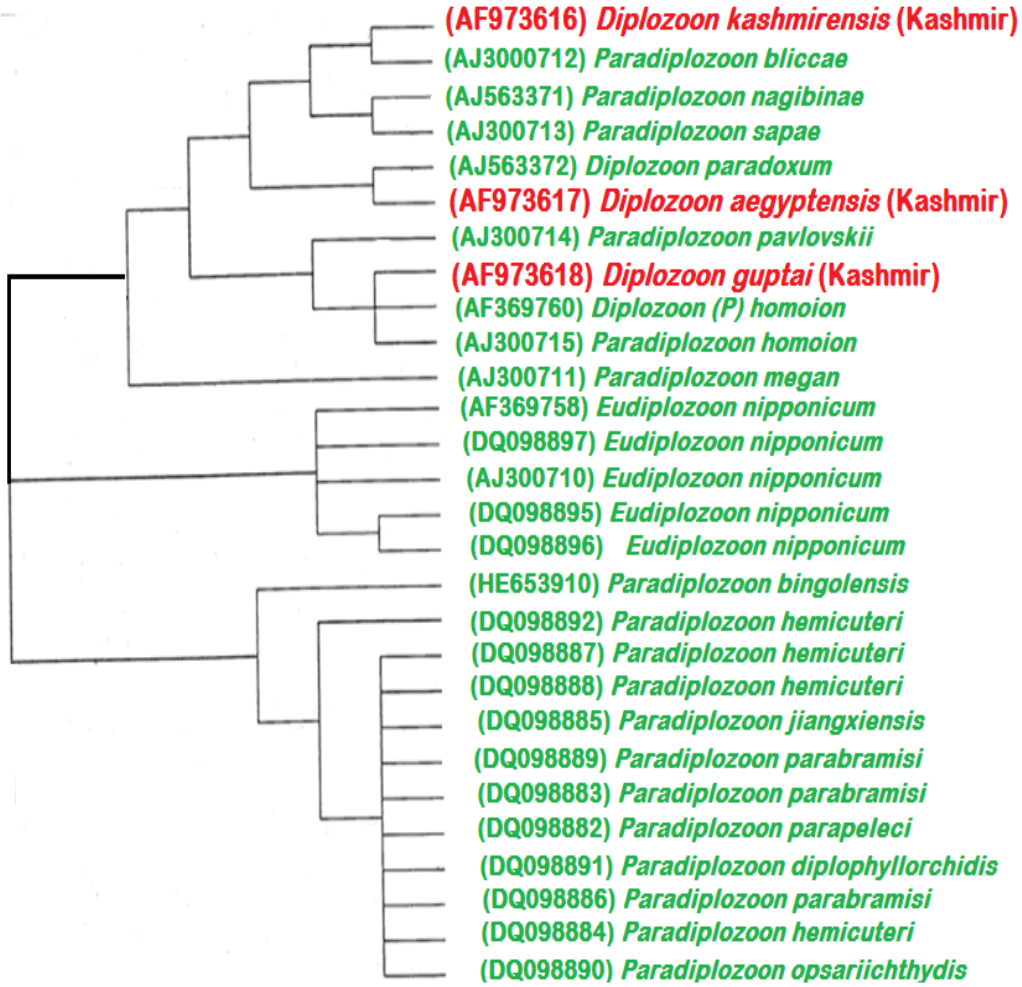


**Fig. 8.** Unrooted bootstrap consensus tree of MP/ML/NJ analysis based on ML tree topology.

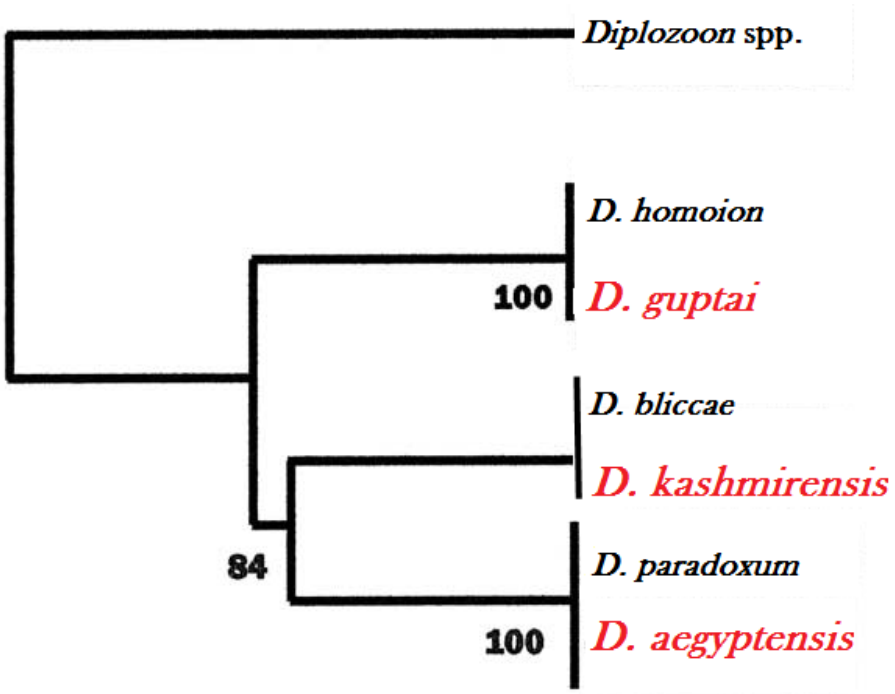


**Fig. 9.** Phylogenetic tree depicting the genetic relationship among three of Diplozoid species by Neighbouring Joining (NJ).

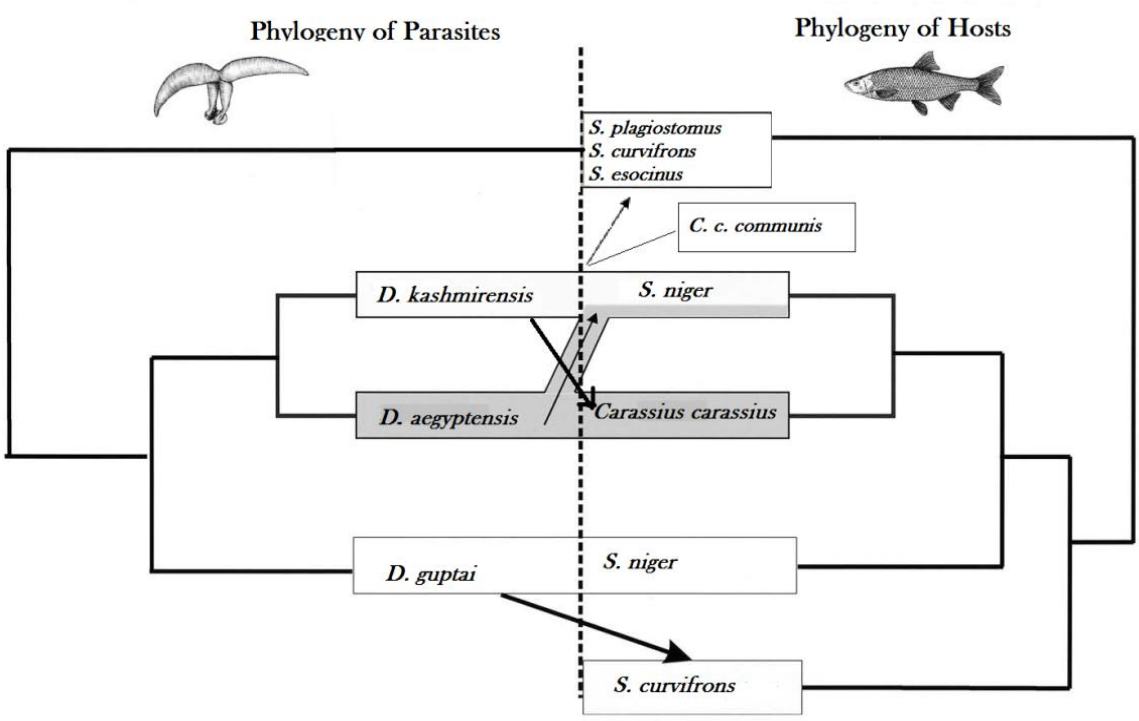


**Fig. 10.** Construction of phylogenetic tree of parasites and hosts showing host parasite relationship of three Diplozoon species in Kashmir.

**Table 3**
Sequences producing significant alignments in *Diplozoon* species (Monogenea).

| Description | Max score | Total score | Query cover | E value | Identity | Accession No. |
|---|---|---|---|---|---|---|
| *Diplozoon bliccae* 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence | 1574 | 1574 | 97% | 0.0 | 93.08% | AF369761.1 |
| *Diplozoon paradoxum* 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence | 1844 | 1844 | 97% | 0.0 | 94.13% | AF369759.1 |
| *Diplozoon homoion* 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence | 1502 | 1502 | 100% | 0.0 | 86.16% | AF369760.1 |
| *Diplozoon* sp. 28S ribosomal RNA, partial sequence | 468 | 468 | 26% | 9e-128 | 82% | AF131717.1 |
| *Paradiplozoon bliccae* partial 5.8S rRNA gene, ITS2, and partial 28S rRNA gene | 1142 | 1142 | 74% | 0.0 | 79% | AJ300712.2 |
| *Paradiplozoon homoion* partial 5.8S rRNA gene, ITS2, and partial 28S rRNA gene | 1092 | 1092 | 76% | 0.0 | 79% | AJ300715.2 |
| *Paradiplozoon sapae* partial 5.8S rRNA gene, ITS2, and partial 28S rRNA gene | 1085 | 1085 | 76% | 0.0 | 79% | AJ300713.2 |
| *Paradiplozoon nagibinae* 5.8S rRNA gene (partial), ITS2 and 28S rRNA gene (partial) | 1035 | 1035 | 76% | 0.0 | 74% | AJ563371.1 |
| *Paradiplozoon pavlovskii* partial 5.8S rRNA gene, ITS2, and partial 28S rRNA gene | 998 | 998 | 76% | 0.0 | 71% | AJ300714.2 |
| *Paradiplozoon megan* 5.8S rRNA gene (partial), 28S rRNA gene (partial) and internal transcribed spacer 2 (ITS2) | 904 | 904 | 74% | 0.0 | 71% | AJ300711.1 |
| *Paradiplozoon sp*. BJVV-2012 genomic DNA containing 5.8S rRNA gene, ITS2 and 28S rRNA gene, isolate RAU:11A | 835 | 835 | 73% | 0.0 | 71% | HF566124.1 |
| *Eudiplozoon nipponicum* 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence | 821 | 821 | 99% | 0.0 | 68% | AF369758.1 |
| *Paradiplozoon* sp. BJVV-2013 genomic DNA containing 5.8S rRNA gene, ITS2 and 28S rRNA gene | 531 | 531 | 72% | 1e-146 | 69% | HG423142.1 |
| *Eudiplozoon nipponicum* isolate DDLiy 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence | 494 | 494 | 77% | 1e-135 | 79% | DQ098897.1 |
| *Eudiplozoon nipponicum* isolate DHonghLiy 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence | 488 | 488 | 77% | 7e-134 | 79% | DQ098896.1 |
| *Eudiplozoon nipponicum* isolate LiyuTxP 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence | 483 | 483 | 77% | 3e-132 | 79% | DQ098895.1 |
| *Eudiplozoon nipponicum* partial 5.8S rRNA gene, ITS2, and 28S rRNA gene | 479 | 479 | 76% | 4e-131 | 79% | AJ300710.2 |
| *Eudiplozoon nipponicum* 28S large subunit ribosomal RNA, partial sequence | 379 | 379 | 26% | 4e-101 | 77% | AF382037.1 |

**Table 4**

Monogenean trematode species used for molecular comparison of ITS rDNA sequences along with their hosts, country and GenBank accession numbers for corresponding sequences (*Query sequence).

| S. No. | Monogenean Species | Host | GenBank Accession No. | Family | Base pair length | Authors | Country | Year |
|---|---|---|---|---|---|---|---|---|
| 1. | *D. kashmirensis* Kaw, 1950* | *Carassius Carassius; Cyprinus carpio cummunis; Schizothorax niger; S. esocinus; S. curvifrons* | AF973616 | Diplozoidea | 873 bp | Present study | India | 2015 |
| 2. | *D. aegyptensis* Fischthal *et* Kuntz, 1963* | *Carassius Carassius; Schizothorax niger;* | AF973617 | Diplozoidea | 1120 bp | Present study | India | 2015 |
| 3. | *D. guptai* Fayaz and Chishti, 2000* | *Schizothorax niger;* | AF973618 | Diplozoidea | 687 bp | Present study | India | 2015 |
| 4. | *D. bliccae* (Glaser, 1965) | *Blicca bjoerkna* | AF369761 | Diplozoidea | 988 bp | Sicard *et al*. | France | 2001 |
| 5. | *D. paradoxum* Nordmann, 1832 | *Abramis brama* | AF369759 and AJ563372 | Diplozoidea | 769 bp | Matejusova | Czech Republic | 2004 |
| 6. | *D. homoion* Bychowsky & Nagibina, 1959 | *Rutilus rutilus, Scardinius erythrophthalmus* | AF369760 | Diplozoidea | 996 bp | Sicard *et al*. | France | 2001 |

**Table 5**
Summary of base pairs and amino acids of *Diplozoon kashmirensis* Kaw, 1950.

| **Length** | **A** | **C** | **G** | **T** | **G+C** |
|---|---|---|---|---|---|
| 873 bp | 177 | 191 | 226 | 279 | 47% |
| Total No. of Amino Acids | | | | | 280 |
| Molecular Weight | | | | | 30827 Da |

**Table 6**
Summary of base pairs and amino acids of Diplozoon aegyptensis Kuntz, 1963.

| Length | A | C | G | T | G+C |
|---|---|---|---|---|---|
| 1120 bp | 237 | 224 | 312 | 345 | 47% |
| Total No. of Amino Acids | | | | | 353 |
| Molecular Weight | | | | | 38825 Da |

**Table 7**
Summary of base pairs and amino acids of Diplozoon guptai Fayaz et Chishti, 2000.

| **Length** | **A** | **C** | **G** | **T** | **G+C** |
|---|---|---|---|---|---|
| 687 bp | 123 | 148 | 188 | 228 | 48% |
| Total No. of Amino Acids | | | | | 219 |
| Molecular Weight | | | | | 24323 Da |

**Table 8**

Pairwise alignments of the 28S rDNA ITS consequences of *Diplozzon kashmirensis* and *Diplozoon bliccae*, numbering refers to ITS sequences.

| | | | |
|---|---|---|---|
| *D. kashmirensis* | 6 | ACTGCCTTGAGCATCGACTTCTTGAACGTAAATTGCGGCATTAGGCTCTGCTGATGCCAC | 65 |
| *D. bliccae* | 6 | GCTGACTTGAGCATCGATTTCTTGAACGTGAATTGCGGCATTACCCTCTAATGATGCCAC | 65 |
| *D. kashmirensis* | 66 | GCCTAGCCGAGTGTCGGCATTAAATCTATCACGACGCTTAATTGGTCGTGGCTTAGTTTG | 125 |
| *D. bliccae* | 66 | GCCTAGCCGAGTATCGGCATTAAATCTAGCACGACGCTTATTTGGTCCTGGCTTAGAAAG | 125 |
| *D. kashmirensis* | 126 | TTGTCAGCCGTCGTGTTGTACT---CAACGTGTTGTTCAGTTGTCAAGTCGACGGTATTA | 185 |
| *D. bliccae* | 126 | TTGTCAGCCGTCGTGTTGTACTTGGCAACGTGTTGTTCTTTTGTCAAGTCGGCGGTATTA | 185 |
| *D. kashmirensis* | 186 | TTGACGCTTGCCAAATGTAATGGAGAGTTTGTATATGC--AATATCTGCCGGTAGCCTGT | 245 |
| *D. bliccae* | 186 | TTGACGCTTGCCAAATGTAATGGAGAGTTTGTATATGCGAAATATCTTCCGGTAGCCTGT | 245 |
| *D. kashmirensis* | 246 | TGGTGTTGGCTACGCTGCCCCGTGTATGGTTTATTTGCATTTTTGTGCATACCGATGGGG | 305 |
| *D. bliccae* | 246 | TGGTGTTGGCTACGCTGCCCCGTGTATTTTTTATTTGCATTTTTGTGCATACCGATGGGG | 305 |
| *D. kashmirensis* | 306 | TGGTTAGCTTCTCGTCATCAGTGCGTGTTTGCCGGTGG-GTCGTGGCGTGGGAATTTCAA | 365 |
| *D. bliccae* | 306 | TGGTTAGCTTCTCGTCAGCAGTGCGTCCTTGCCGGTGGTGTCGTGGAATGGGAATTTCAA | 365 |
| *D. kashmirensis* | 366 | TAAGCATTTCTGAATGGTAATTGTGAAATTGTCAT---ATGTGCTGTTCTCTTGAGCCTT | 425 |
| *D. bliccae* | 366 | TAAGCATTTCTGAATCCTAATTGTGAAATTGTCATTTTATGTGCTGTTCTCTTGAGCCGC | 425 |
| *D. kashmirensis* | 426 | TTGGCCCACGGGTTGTGCGGTGACCAGTGTTGCTTTGAATGCGAGCGCATGCATGCCAGG | 485 |
| *D. bliccae* | 426 | ACGGCCCACTTATTGTGCGATGACCAGTGACGCTTTGAATGCGAGTGCATGCATGCCAGG | 485 |
| *D. kashmirensis* | 486 | TCGCAGCCTATTTGTGATCGCGAC-GTGCTTTGCTTGTGTTCTGCGTTTAATTTTTGTCA | 545 |
| *D. bliccae* | 486 | TCTCAGCCTATTTGTGATCGCGACAGTGCTTTGCTTGTGTTCTGCGTTTAATTTTTGTCA | 545 |
| *D. kashmirensis* | 546 | CTGTTTCTTGCGAATGAGCGAGTCTGGCCCGAGACGAGATTATGTGCCCATGTCGTGCTG | 605 |
| *D. bliccae* | 546 | CTGTTTCCCGCGAATGAGCGAGTCTGGCCCGAGACGAGAGCATGTGCCCATGTCGTGCTG | 605 |
| *D. kashmirensis* | 606 | TGCAGACATTACTACTCCATTGGTCGCTAAGTGCATATCGGTGTC--CCGTATTCTACTG | 665 |
| *D. bliccae* | 606 | TGCAGACATTACTACTCCATTCTTCGCTAAGTGTGTATCGGTGTCACCCGTATTTTACTG | 665 |
| *D. kashmirensis* | 666 | TACTGCTGTGGTGTGTGCACCTGACCTCGGATTAGGCGTGATTACCCGCTGAACTTAAGC | 725 |
| *D. bliccae* | 666 | TACTTCTGTGGTGTATGCACCTGACCAAGGATTAGGCGTGATCACCCGCTGAGCTTAAGC | 725 |

| | | | | |
|---|---|---|---|---|
| *D. kashmirensis* | 726 | ATATCAATAAGCGGAGGAAAAGAAACTAACCAGGATTCCCTT-GTAACGGCGAGTGAACA | ‖‖‖‖‖‖‖ | 785 |
| *D. bliccae* | 726 | ATATCAATGGGCGGAGGAAAAGAAACTAACCACTATTCCCTTAGTAACGTCGAGTGAACA | | 785 |
| *D. kashmirensis* | 786 | GGGATTAGCCCAGCACCGAAGCTGCGGTC--TTGGCCGTTCGGCAATGTGGTGTTTAGGT | ‖‖‖‖‖‖‖ | 845 |
| *D. bliccae* | 786 | CCGATTAGCAAAGCACCGAAGCTGCGGTCTTTTGGCCGTTCGGCAATCCGGTGTTTAGGT | | 845 |
| *D. kashmirensis* | 846 | TGGCATACTCAGGCGATGTACTGTGTAG | | 873 |
| *D. bliccae* | 846 | TATCATACTCAGGCGATGTACTGTGCCC | | 873 |

**Table 9**

Pairwise alignment of the 28S rDNA ITS consequences of *Diplozoon aegyptensis* and *Diplozoon paradoxum*, numbering refers to ITS sequences.

| | | | |
|---|---|---|---|
| *D. aegyptensis* | 1 | TGCAAACTGCCTTGAGCATCGACTTCTTGAACGTAAATTGCGGCATTAGGCTCTG-CTGA | 59 |
| *D. paradoxum* | 4 | TGCAAACTGCCTTGAGCCTCGACTTCCCGAACGTAAATTACGGCATTAGGCTCTGCCTGA | 63 |
| *D. aegyptensis* | 60 | TGCCACGCCTAGCCGAGTGTCGGCATTAAATCTATCACGACGCTTAATTGGTCGTGGCTT | 119 |
| *D. paradoxum* | 64 | TGCCCGACCTAGCCGAGTGTCGGCATTAAATCTATCACGACATAATATTGGTCGTGGCTT | 123 |
| *D. aegyptensis* | 120 | AGTTTGTTGTCAGCCGTCGTGTTGTACTTGGCAACGTGTTGTTCAGTTGTCAAGTCGACG | 179 |
| *D. paradoxum* | 124 | AGTTTGTTAAAAGCCGTCGTGTTGTACTTAACAACGTGTTGTTCAGTTGTCAAGTAGACG | 183 |
| *D. aegyptensis* | 180 | GTATTATTGACGCTTGCCAAATGTAATGGAGAGTTTGTATATGCGAAATATCTGCCGGTA | 239 |
| *D. paradoxum* | 184 | GTATTATTGACGCTTGCCAAATGTAATGGAGAGTTAG-NDATGCGAAATATCCGCTGGTA | 242 |
| *D. aegyptensis* | 240 | GCCTGTTGGTGTTGGCTACGCTGCCCCGTGTATGGTTTATTTGCATTTTTGTGCATACCG | 299 |
| *D. paradoxum* | 243 | GCCTGTTGGTGTTGGCAACGCTGTCCCGTGTATGGTTTACTTGCATTTTTGTGCATACCG | 302 |
| *D. aegyptensis* | 300 | AT-GGGGTGGTTAGCTTCTCGTCATCAGTGCGTGTTTGCCGGTGGTGT----C-GTGGCG | 353 |
| *D. paradoxum* | 303 | ATGGGGGCGGTTAGCTTCGCGTCATCAGAGCGTGTTTGCCGGTGGTGTATTGCAGTGGCG | 362 |
| *D. aegyptensis* | 354 | TGGGAATTTCAATAAGCATTTCTGAATGGTAATTGTGAAATTGTCATTTTATGTGCTGTT | 413 |
| *D. paradoxum* | 363 | TGGGAATTTCAATGAGCATTTGTGAATGGTAATTGTTAAATTGCCATTTTATGTGCTGTT | 422 |
| *D. aegyptensis* | 414 | CTCTTGAGCCTTTTGGCCCACGGGTTGTGCGGTGACCAGTGTTGCTTTGAATGCGAGCGC | 473 |
| *D. paradoxum* | 423 | CTCTTGAGCCTTTTGGCTTTCGGGTTGTGCGGTGACCAGTGTTGCTTTGAATGCGTGCGC | 482 |

| | | | |
|---|---|---|---|
| *D. aegyptensis* | 474 | ATGCATGCCAGGTCGCAGCCTATTTGTGATCGCGACAGTGCTTTGCTTGTGTTCTGCGTT | 533 |
| *D. paradoxum* | 483 | ATGCATGCCAGGTCGCAGCCTA-TTGTGATCGCGACAGTGCTTTGCTTGTGTTCTGCGTT | 541 |
| *D. aegyptensis* | 534 | TAATTTTTGTCACTGTTTCTTGCGAATGAGCGAGTCTGGCCCGAGACGAGATTATGTGCC | 593 |
| *D. paradoxum* | 542 | TAATTTTTGTCACTGCCGCTTGCGTATGTGCGAGTGTGACCCGAGATGAGATTGTGTGCC | 601 |
| *D. aegyptensis* | 594 | CATGTCGTGCTGTGCAGACATTACTACTCCATTGGTCGCTAAGTGCATATCGGTGTCACC | 653 |
| *D. paradoxum* | 602 | CATGTCATGCTGTGCTGACATTACTACTCCACTGGTCGCTAAGTGCATGTCGGTGTCATC | 661 |
| *D. aegyptensis* | 654 | CGTATTCTACTGTACTGCTGTG--GTGTGTGCACCTGACCTCGGATTAGGCGTGATTACC | 711 |
| *D. paradoxum* | 662 | AGTATTCTACTGTACTGCTGTGTTGTGTGTGCACCTGACCTCGGATTAGGCGTGATTACC | 721 |
| *D. aegyptensis* | 712 | CGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACCAGGATTCCCTTAGTA | 771 |
| *D. paradoxum* | 722 | CGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACCAGGATTCCCTTAGTA | 781 |
| *D. aegyptensis* | 772 | ACGGCGAGTGAACAGGGATTAGCCCAGCACCGAAGCTGCGGTCTTTTGGCCGTTCGGCAA | 831 |
| *D. paradoxum* | 782 | ACGGCGAG----CAGGGATTAGCCCAGCACCGAAGCTGCGGTCTTTTGGCCGTTCGGCAA | 841 |
| *D. aegyptensis* | 832 | TGTGGTGTTTAGGTTGGCATACTCAGGCGATGTACTGTGCTAAGTCCATTCATGAATATG | 891 |
| *D. paradoxum* | 842 | TGTGGTGTTTAGGTTGGCATACTCAGGCGATGTACTGTGCTAAGTCCATTCATGAATATG | 901 |
| *D. aegyptensis* | 892 | GCTAGCTATCTGGCCCAGAGAGGGTGAAAGGCCCGTGAGCATAGTGCGTCGTTCTGTCTT | 951 |
| *D. paradoxum* | 902 | GCTAGCTATCTGGCCCAGAGAGGGTGAAAGGCCCGTGAGCATAGTACGTTGTTCTGTCTT | 961 |
| *D. aegyptensis* | 952 | AGTCAACCGTTGAGTCGGGTTGTTTAGGAATGCAGCC | 988 |
| *D. paradoxum* | 962 | AGCCAACCGTTGAGTCGGGTTGTTTAGTAATGCAGCA | 998 |

**Table 10**
Pairwise alignments of the 28s rDNA ITS consequences of *Diplozzon guptai* and *Diplozoon homoion*, numbering refers to ITS sequences.

| | | | |
|---|---|---|---|
| *D. guptai* | 9 | ACTGCCTTGAGCATCGACTTCT--AACGTAAATCGCGGTATTAGGCTCTGCCTGATGCCA | 68 |
| *D. homoion* | 6 | ACTGACTTGAGCATCGATTTCTTGAACGTGAATTGCGGCATTACCCTCT-AATGATGCCA | 64 |
| *D. guptai* | 69 | CGCCTAGCCGAGTGTTGGCATTATATCTATCACGACGCTTAATTGGTCGTGGCTTAGTTT | 128 |
| *D. homoion* | 65 | CGCCTAGCCGAGTATCGGCATTAAATCTAGCACGACGCTTATTTGGTCCTGGCTTAGAAA | 124 |
| *D. guptai* | 129 | GTTGTCAGCCGTCGTGTTTTACTTTGCAACGTGTTGCTCAGTTGTAAAGTCGACGGTATT | 188 |
| *D. homoion* | 125 | GTTGTCAGCCGTCGTGTTGTACTTGGCAACGTGTTGTTCTGTTGTCAAGTCGGCGGTATT | 184 |
| *D. guptai* | 189 | ATTGACGCTTGCCAAATGTAATGGAGAGTGTGTATATGCGAAATTTCTGCCGG-AGCCTG | 248 |
| *D. homoion* | 185 | ATTGACGCTTGCCAAATGTAATGGAGAGTTTGTATATGCGAAATATCTTCCGGTAGCCTG | 244 |
| *D. guptai* | 249 | TTGGCGTTGGCGACGCTGCCCCGTGTATGGTTTACTTGCATTTTTGTGCATACCGATTGG | 308 |
| *D. homoion* | 245 | TTGGTGTTGGCTACGCTGCCCCGTGTATTTTTTATTTGCATTTTTGTGCATACCGA-TGG | 303 |
| *D. guptai* | 309 | GGCGGTTAGCTTGTCGTCATCAGTGCGTGTTTGCCGGTGGTGATTTGTGGTGGCGTGGGA | 368 |
| *D. homoion* | 304 | GGTGGTTAGCTTCTCGTCAGCAGTGCGTCCTTGCCGGTGG-----TGTCGTGGAATGGGA | 358 |
| *D. guptai* | 369 | ATTTCAATAAGCATTACTGAATGGTAATTAATAAATTGCCATTATATATGCTGTTCTCTT | 428 |
| *D. homoion* | 359 | ATTTCAATAAGCATTTCTGAATCCTAATTGTGAAATTGTCATTTTATGTGCTGTTCTCTT | 418 |
| *D. guptai* | 429 | GAGCCTTTTGGCCCACGGGTTGTGCGGTGACCAGTGTTGCTTTGAATGCGTGCGCATGCA | 488 |
| *D. homoion* | 419 | GAGCCGCATGGCCCACTTGTTGTGCGATGACCAGTGACGCTTTGAATGCGAGTGCATGCA | 478 |
| *D. guptai* | 489 | TGCCAGGTCGCAGCCTA-TTGTGATCGCGACAGTGCTTTGCTTGTGTTCTGCGTTTATTT | 547 |
| *D. homoion* | 479 | TGCCAGGTCTCAGCCTATTTGTGATCGCGACAGTGCTTTGCTTGTGTTCTGCGT--AATT | 538 |
| *D. guptai* | 548 | GTTGTCACTGCTACTTGCATATGTGCGAGTGTGTACCCGGAATGAGATTTTGTGCCCATG | 607 |
| *D. homoion* | 539 | TTTGTCACTGTTTCCCGCGAATGAGCGAGTCTGG-CCCGAGACGAGAGCATGTGCCCATG | 597 |
| *D. guptai* | 608 | TCATGCTGTGCTGACATTACTTCTCCACTGGTCGATAAGTGCATGTCGGTGTCACCAGTA | 667 |
| *D. homoion* | 598 | TCGTGCTGTGCAGACATTACTACTCCATTCTTCGCTAAGTGTGTATCGG-GTCACCCGTA | 657 |
| *D. guptai* | 668 | CTTTGCTGTA-TT--GTG-T | 687 |
| *D. homoion* | 658 | TTTTACTGTACTTCTGTGGT | 677 |

Table 8 shows that Diplozoon kashmirensis having GenBank accession number AF973616 mostly resembles with Diplozoon bliccae with an accession number AF369761.1. Out of 867 base pairs of Diplozoon kashmirensis, 807 bp match with that of Diplozoon bliccae i.e., 93.08% similarity with 15 gaps (1.73%).

From the Table 9 it is clear that Diplozoon aegyptensis having GenBank accession number AF973617 shows 94.13% similarity with Diplozoon paradoxum with an accession number AF369759.1. Out of 988 base pairs of Diplozoon aegyptensis, 930 bp match with that of Diplozoon paradoxum with 11 gaps (1.11%).

The present observation shows that Diplozoon guptai having GenBank accession number AF973618 shows 86.16% similarity with that of Diplozoon homoion having GeneBank accession mnumber AF369760.1 (Table 10). 585 bp of Diplozoon guptai matches with Diplozoon homoion with 15 gaps, out of total 679 base pairs.

**3. Discussion**

The rDNA second internal transcribed spacer (ITS2) was amplified using primers Cer5.8S2249 and Cer28S3116 (Sicard et al; 2001) for 3 species of diplozoids. Analysis of the ITS2 region following sequencing clearly allowed us discrimination at the species level and produced the same results as species identification made by using morphological structures. During the present study it was observed that the alignment of nucleotide sequences with those of other Diplozoon species of D. bliccae, D. paradoxum and D. homoion (Sicard et al., 2001; Mutejusova et al., 2001), clearly revealed the boundaries of the 5.8S and 28S rDNA genes, as the sequences in these species closely resembles to those of D. kashmirensis, D. aegyptensis and D. guptai. As noted in comparison of ITS2 sequences of Monogenean species, the first part of the ITS2 is also highly conserved, with only 6 variable sites in the first 65 nucleotides of the diplozoid sequences.

Species discrimination of diplozoids based on the shape of clamp sclerites and the length of the central hook can be difficult because of similarities in the shape of certain sclerites and overlapping ranges of central hook measurements. The PCR product of 3 species of diplozoids D. kashmirensis, D. aegyptensis and D. guptai were clearly discriminated on the basis of nucleotide sequences which were different in their length of base pairs. The length of the PCR product could be useful to distinguish Diplozoids from the genus Eudiplozoon and Paradiplozoon from other Diplozoids (Matejusova et al., 2001). Length differences in the ITS2 have also been recorded in the genus Gyrodactylus (Matejusova et al., 2001) but are not generally as large as those found in the ITS1 region of Lamellodiscus and Gyrodactylus (Cable et al., 1999; Desdevises et al., 2000; Matejusova et al., 2001). During the present study there are length difference of PCR products of three Diplozoon species i.e., D. kashmirensis contains 873 bp; D. aegyptensis contains 1120 bp and D. guptai contains 687 bp of 28S rDNA genes, so on the basis of length of base pairs the three diplozoid species can be discriminated. ITS region have been found to be useful species markers for monogenean parasites (Cunningham, 1997; Matejusova et al., 2001; Huyse and Volckaert, 2002; Zietara et al., 2002; Simkova et al., 2006) so, this method was performed to distinguish the diplozoid species. During the present study, the intraspecific variations within diplozoid species were studied and differences were detected in the ITS regions, but Matejusova et al., 2001 studied that ITS region lacks intraspecific variation in groups of Monogenea which is due to the same species recovered from different hosts.

Diplozoids are generally considered parasites of Cyprinid species but the host specificity differs and relates to geographical origin. In Eurasia, diplozoid occurrence is restricted to host fishes from the Cyprinidae and Perciformes families (Khotenovsky, 1985; Mutejusova et al., 2001, 2002, 2004; Yildirim et al., 2010). However, in Africa they also parasitize members of the Characidae (Khotenovsky, 1985; Lambert and Le Brun, 1988). All diplozoid species described in the present study are also host specific. ML, MP and NJ trees showed that D. kashmirensis; D. aegyptensis and D. guptai are closely related species, and this mirrors the close relationship of their hosts, thus all of these species are found in cyprinids from the same genus Schizothorax. These species have been described morphologically based on clamp shape, total body length, sucker, and pharynx length (Bakshi, 1999; Fayaz and Chishti, 1999). The present observations on molecular characterization demonstrate sufficient genetic variations between parasites from different hosts to confirm the validity of these species and that they appear to be host specific, as are many monogenean parasites. It may be speculated that the similarity of these species is a result of a relatively recent divergence of one from the other following a host-switching event. An important observation during the present study has been noticed that Schizothorax niger is infected by all the three species of Diplozoidae: D. kashmirensis; D. aegyptensis and D. guptai, but on all six fishes collected, simultaneous parasitism by all the parasite species was never observed. Two types of factors can be involved in the constitution of such a host-parasite system. (a) Competition hypothesis: the installation of a first Diplozoon species

prevents any other species from settling on the same gill. (b) Since natural hybridization has been reported between the two fishes, the introgression of genes from Carassius carassius into the genome of S. niger allows a host capture of the latter by D. aegyptensis and D. guptai but excludes the infestation by its natural parasite D. kashmirensis. To test this, a through characterization of the host species will be required.

**4. Conclusion**

In conclusion, the present study has confirmed the existence of 3 species of diplozoids from 6 species of cyprinid fishes from the water bodies of Kashmir valley. All the species were clearly distinguished by differences in nucleic acid sequences within the second ribosomal DNA internal transcribed spacer region (ITS2). Analysis of additional specimens from different cyprinid hosts by molecular methods may be helpful to clarify the systematics of this fascinating family Diplozoidae.

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